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HIGH-THROUGHPUT KINETIC CHARACTERIZATION OF RICIN TOXIN B CHAIN AND OVALBUMIN ANTIBODIES USING SURFACE PLASMON RESONANCE

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

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14. ABSTRACT

Hybridoma cell lines were developed against ricin toxin B (RTB) chain and ovalbumin. These cells were then grown in culture and purified for characterization and possible placement in the Critical Reagents Program repository. From the cell culture medium, antibodies were purified using protein A purification, desalted in 1× phosphate buffered saline using Sephadex G-25 columns, and then filtered with a 0.2 µm filter. Twenty-seven purified clones were characterized against their respective immunogens using a Bio-Rad ProteOn XPR36 high-throughput surface plasmon resonance instrument. RTB clones were also characterized against ricin holotoxin. Using an indirect assay format, we estimated kinetic data and toxin reactivity in 14 h. Data were collected and analyzed using Scrubber Pro software. Two clones were found to bind ricin holotoxin in addition to the RTB. Kinetic constants for the ovalbumin clones were also obtained. In the future, this high-throughput screening method can be applied to other purified antibody clones to rank their utility in downstream field assay applications.

15. SUBJECT TERMS

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Surface plasmon resonance	Label-free biosensor analysis	Kinetics	

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PREFACE

The work described in this report was started in July 2007 and completed in August 2010.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for the purposes of advertisement.

In conducting the research described in this report, the investigators adhered to SOP RNB-206.[*] Appropriate safety precautions and personal protective equipment were used to perform these experiments.

This report has been approved for public release.

Acknowledgments

The authors wish to express their thanks to William Edwards and Matt Brown (U.S. Army Edgewood Chemical Biological Center student contractors) for their specialized help; and Vanessa Funk, Bonnie Woffenden, and Melissa Dixon (Science and Technology Corporation, Edgewood, MD) for their help in developing these antibodies.

[*] Calm, A. Microbiological and Molecular Operations; SOP RNB-206; U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD, 2012.

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HIGH-THROUGHPUT KINETIC CHARACTERIZATION OF RICIN TOXIN B CHAIN AND OVALBUMIN ANTIBODIES USING SURFACE PLASMON RESONANCE

1. INTRODUCTION

Antibodies are reagents commonly used in chemical and biological detection assays. They are relatively easy to obtain and produce, and they can be highly sensitive and specific. A standard method of producing antibodies is by hybridoma development, whereby a protein of interest is injected into a mouse to produce an immune response. Over time, the mouse develops antibodies against the antigen. The spleen of the mouse is harvested and fused with myeloma cells to produce an immortalized cell line that excretes antibodies specific to the immunogen. These antibodies are purified in their monoclonal form so that they can be characterized, ranked, and moved into field assays.

With the ever present threat of biological and chemical warfare, the need for antibodies against chemical and biological threats is critical. Recent and historical events have shown that the toxic ribosome inactivating protein, *Ricinus communis*, or ricin toxin, which is derived from the castor bean, is a prime candidate for biological warfare. Ricin is composed of two subunits, the A and B chains. The ricin B chain allows the toxin entry into the cell. When the ricin B chain is attached to the ricin A chain, the A chain inactivates ribosomes. Each subunit by itself is harmless, but as a whole, ricin is toxic, even lethal. Used as a weapon in 1978 against Bulgarian dissident Georgi Markov, ricin proved to be a lethal weapon. Again in 2008, ricin was shown to be lethal when Roger von Bergendorff was hospitalized and then arrested after inhaling the ricin that he was attempting to purify and mass produce. These incidents, along with speculations and the known ease of production and purification of ricin toxin, have necessitated the development of antibodies against ricin with the intention of using them in rapid detection assays.

In addition to antibodies against toxins, there is also a need for antibodies against biological simulants. Ovalbumin (OVA) from chicken egg is a simulant of interest to the defense community. With funding from the Critical Reagents Program (Frederick, MD), hybridomas were created to generate antibodies against the ricin B chain and OVA. The following experiment describes the screening of these particular antibodies, using high-throughput surface plasmon resonance (SPR) technology (Biosensing Instrument, Tempe, AZ).

2. EXPERIMENTAL PROCEDURES AND MATERIALS

The three processes used in the screening for ricin antibodies and the materials used for those processes are listed below:

- Purification of clones

The following materials were used in this process:

- Protein A XK 16/15 (30 mL) column
- Desalting Sephadex G-25 (Sigma-Aldrich, Rockville, MD) Fine XK26/12 (63 mL)
- phosphate buffered saline (PBS) buffer at pH 7.2
- 50 mM sodium citrate buffer pH 3.0 (elution buffer)

- Chip immobilization

The following materials were used in this process:

- Bio-Rad (Bio-Rad Laboratories Inc. Products, Irvine, CA) ProteOn XPR36
- Bio-Rad ProteOn PBS/tween (PBST) 176-2720
- Bio-Rad ProteOn immobilization buffer kit 176-2110
- Bio-Rad ProteOn amine coupling kit (EDC, NHS, Ethanolamine) 176-2410
- Bio-Rad ProteOn GLC sensor chip176-5011

- Kinetic experiment

The following materials were used in this process:

- Bio-Rad ProteOn XPR36
- Bio-Rad ProteOn PBST 176-2720
- Bio-Rad ProteOn GLM sensor chip 176-5012
- Purified antibodies
- Ricin holotoxin
- Bio-Rad ProteOn 0.85% phosphoric acid solution 176-2260
- Scrubber Pro software (Biologic Software, Campbell, Australia)
- Nanodrop 1000 (ThermoFisher Scientific, Wilmington, DE)

3. METHODS

SPR technology is used to measure protein:protein interactions. An SPR chip, made up of a prism attached to a thin piece of gold with a layer of alginate on it, is inserted into an SPR instrument. A photon of light is shone through the prism, and the angle of reflected light is measured and recorded. When mass is added to the alginate layer on the surface of the gold, the angle of reflection changes. The optics of this instrument are sensitive to refractive index changes caused by buffer switching and the addition of mass. An antigen, referred to as the ligand, is typically covalently coupled to the dextran surface. Then, an analyte reacts with the ligand. The SPR instrument is used to measure the binding rate of the analyte to the ligand (k_a) and the rate that the complex dissociates (k_d). These measurements are used to determine the kinetic constant of the interaction (K_D). The Bio-Rad Proteon XPR36 instrument is unique in that it has 6 ligand channels and 6 analyte channels, allowing up to 36 interactions to be analyzed

simultaneously. Up to five ligands and five analytes with a buffer blank in each group can be measured in one experiment. The surface is regenerated by stripping the analytes off the ligand with a harsh salt or acid solution. The same surface can be analyzed again with a new injection of analyte.

3.1 Purification of Clones

To screen the anti-ricin B and anti-OVA clones, the clones were purified using protein A and quantified using a Nanodrop 1000 to measure the protein A280.

3.2 Chip Immobilization

A Bio-Rad ProteOn GLM sensor chip was preconditioned using 0.5% sodium dodecyl sulfate, 100 mM sodium hydroxide, and 10 mM hydrochloric acid. The chip was immobilized using Bio-Rad amine-coupling chemistry. Using 675 µL of each, EDC (ProteOn Amine Coupling Kit 176-2410) was mixed with NHS (ProteOn Amine Coupling Kit 176-2410) to make a 50% solution. Fifty micrograms per milliliter of Sigma rabbit anti-mouse antibody was tethered to the chip at a flow rate of 30 µL/min for 5 min in pH 4.5 sodium acetate buffer with 0.075% Tween 20 in the vertical direction. Ethanolamine was flowed over the surface of the chip for 5 min at a flow rate of 30 µL/min to deactivate it. The chip was then normalized with 50% glycerol.

3.3 Kinetic Analysis

The surface of the chip was stabilized with a vertical injection of PBST (ProteOn PBST 176-2720), and then, each antibody was captured in the horizontal direction, run neat (see mAb concentration table, Appendix A) in 1× PBS at a flow rate of 30 µL/min for 300 s. Five antibodies were captured with each ligand injection, and a PBS blank injection was in the sixth ligand channel.

The surface was stabilized again with a horizontal injection of PBST at a flow rate of 100 µL/min for 1 min. Analyte was injected horizontally at 100 µL/min for 180 s followed by a 600 s dissociation period. For the anti-ricin toxin B (RTB) clones, both the ricin B chain and ricin holotoxin were used as analytes. Ricin B was used at concentrations of 250, 83, 27.8, 9.3, and 3.1 nM. Ricin holotoxin was used at a concentration of 500, 167, 56, 18.5, and 6.2 nM. For the anti-OVA clones, OVA was used as the analyte at concentrations of 1 µM and 333, 111, 37, and 12.3 nM. The surface of the chip was regenerated with 0.85% phosphoric acid for 18 s at a flow rate of 100 µL/min. Data was exported and analyzed using the Scrubber Pro software.

4. RESULTS

4.1 RTB and OVA Analyte Analysis

For antibody capture levels, refer to Appendix A. Analyte sensorgrams were fit in the Scrubber Pro software using a Langmuir 1:1 model (see Appendix B for OVA clones and

Appendix C for RTB clones). The Scrubber Pro software was also useful in determining the kinetic constants for each clone that was able to bind to its respective antigen, ricin B chain, or OVA (Table). The resulting K_D is indicative of how tightly each clone was able to bind to each antigen.

Kinetic constants are listed based on a Langmuir 1:1 model for each clone. Data highlighted in yellow indicates clones that bind ricin holotoxin in addition to the ricin B chain. See Appendices A and B for sensorgram fits and residuals.

Table. Kinetic Constants

Clone Name	Antigen	k_d (s^{-1})	k_a($M^{-1}s^{-1}$)	K_D
P8G9F10	OVA	2.08E-04	1.12E+05	1.85 nM
P8G9G7	OVA	1.53E-04	1.18E+05	1.3 nM
P8G9C11	OVA	1.19E-04	1.36E+05	877 pM
P8G9B10	OVA	1.33E-04	1.50E+05	1.02 nM
P8G9F2	OVA	1.40E-04	1.25E+05	1.12 nM
P8G9A9	OVA	1.23E-04	1.50E+05	816 pM
P8G9G6	OVA	1.41E-04	1.52E+05	928 pM
P8G9D10	OVA	1.71E-04	1.73E+05	995 pM
P8G9H1	OVA	7.93E-05	1.82E+05	437 pM
P8G9C2	OVA	1.57E-04	1.80E+05	875 pM
P8G9C10	OVA	1.60E-04	1.25E+05	1.28 nM
P8G9C9	OVA	1.26E-04	1.23E+05	1.03 nM
P8G9F10	OVA	1.67E-04	1.30E+05	1.28 nM
P8G9H12	OVA	1.27E-04	1.39E+05	911 pM
P8G9G10	OVA	1.51E-04	1.40E+05	1.08 nM
P6H8C6	OVA	1.60E-04	3.39E+05	472 pM
P6H8H5	OVA	2.73E-04	5.65E+05	483 pM
P8C4H10	OVA	9.72E-04	2.46E+04	39.5 nM
P8C4H9	OVA	1.01E-03	2.44E+04	41.6 nM
P1D10B9	Ricin B	3.04E-05	1.73E+04	1.75 nM
P1D10D12	Ricin B	5.39E-05	2.73E+04	1.97 nM
P1D10E12	Ricin B	2.75E-05	3.47E+04	793 pM
P6A7D12	Ricin B	7.98E-05	1.65E+04	4.83 nM
P4A8A12	Ricin B	8.68E-05	8.97E+05	96.8 pM
P4A8G5	Ricin B	2.40E-04	2.63E+05	913 pM
P4A8G5	Ricin Holotoxin	4.40E-04	4.92E+04	8.93 nM
P3F4E4	Ricin B	2.70E-05	3.65E+04	733 pM
P1B4B8	Ricin B	2.53E-04	2.16E+05	1.17 nM
P1B4B8	Ricin Holotoxin	3.94E-04	1.00E+05	3.93nM
P4A8G9	Ricin B	1.23E-04	3.16E+04	3.9 nM
P1D10B11	Ricin B	1.00E-05	3.25E+04	322 pM
P3F4F7	Ricin B	9.01E-05	2.51E+04	3.59 nM
P1B7C12	Ricin B	3.78E-04	1.75E+05	2.16 nM
P1B7C2	Ricin B	2.79E-04	2.64E+05	1.06 nM
P1B7B12	Ricin B	3.01E-04	2.43E+05	1.24 nM

4.2 Ricin Holotoxin Analyte Analysis

After running the ricin B chain and OVA with each clone, as part of the same experiment, ricin holotoxin was tested with each of the anti-ricin B chain clones. Two of the antibodies, P4A8G5 and P1B4B8, interacted with ricin holotoxin (Figure 1). The association, dissociation, and kinetic constants for the clones that interacted with ricin are listed in the Table. These clones can be candidates for assay development.

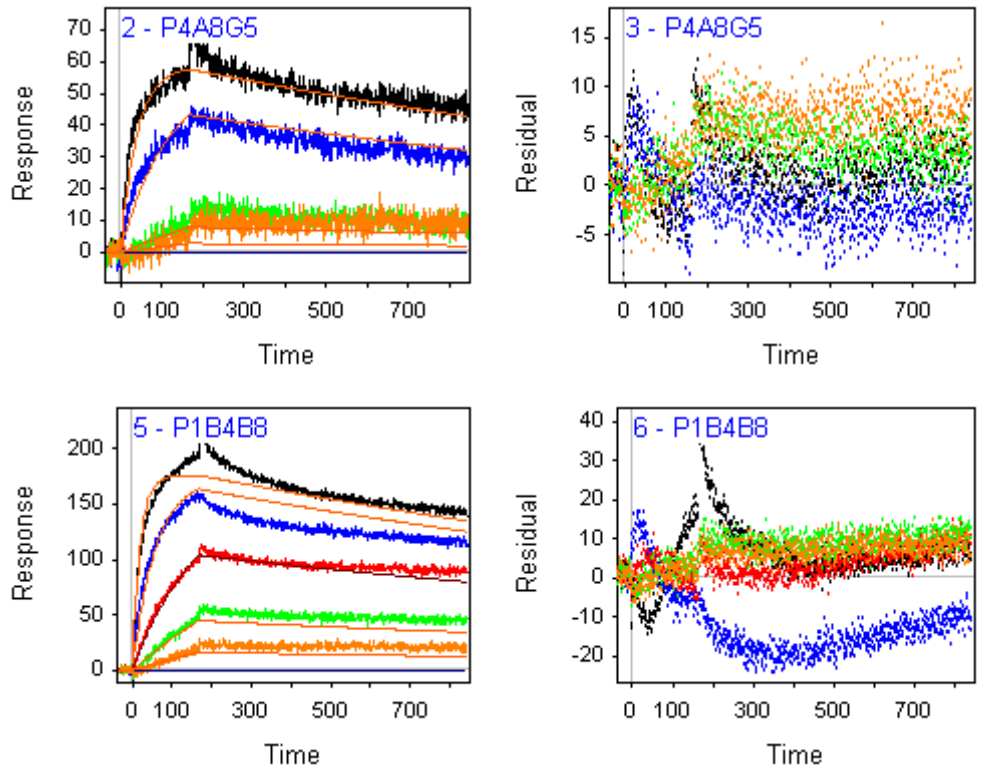

Figure 1. Ricin holotoxin binding P4A8G5 and P1B4B8. Anti-ricin B chain clones, P4A8G5 and P1B4B8, were captured on an anti-mouse chip. The clones interacted with ricin holotoxin using SPR. Analyte sensorgrams were exported into the Scrubber Pro software and analyzed using a Langmuir 1:1 fit. Sensorgrams with overlaid fits and residuals are shown here.

4.3 Kinetic Constant Relative Comparison

For all of the antibodies, the log of the dissociation constants was then plotted with the log of the association constants to show the relative affinities (Figures 2 and 3). The data points are representative of actual K_D values. Most of the kinetic constants fall very close to one another for the OVA clones, but there is more diversity in the RTB dataset.

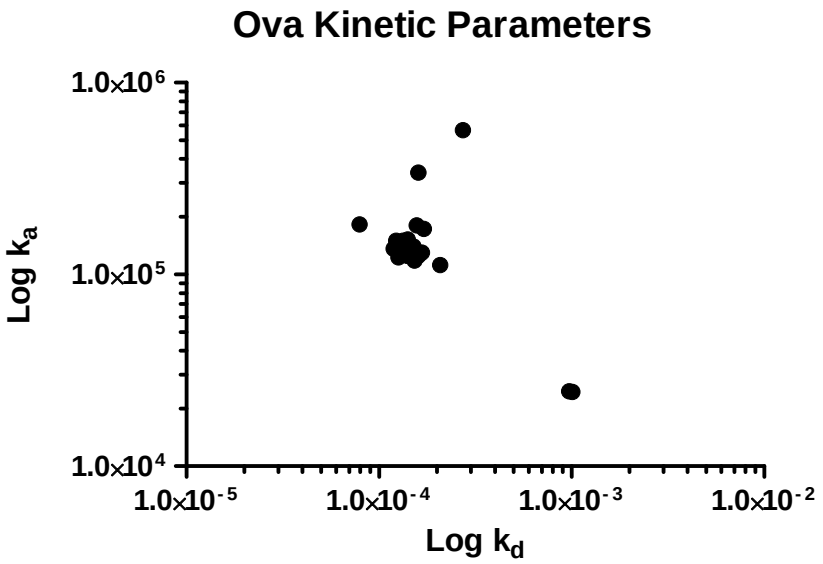

Figure 2. OVA kinetic parameter analysis. Scatter plot of the log of the on (k_a) and off (k_d) rates for the ovalbumin clones.

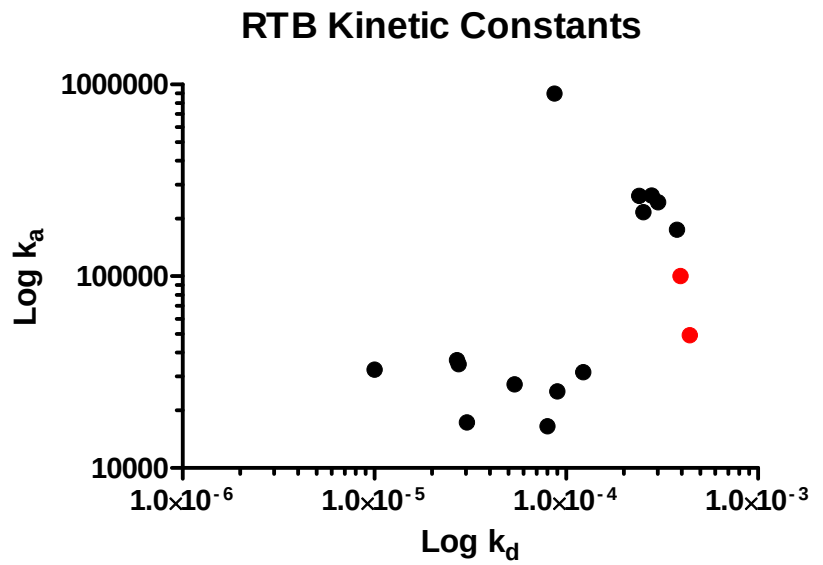

Figure 3. RTB kinetic constants. Scatter plot of the log of the on (k_a) and off (k_d) rates for the RTB clones. The clones that recognize ricin holotoxin are indicated in red.

5. CONCLUSIONS

5.1 Experimental Conclusions

This method of hybridoma production to generate antibodies against the biosimulant OVA and the biothreat ricin was successful. Using SPR, we were able to demonstrate a rapid method for estimating kinetic binding constants for all of the clones that were considered. While only two of the ricin clones bound whole toxin, the majority of the clones responded well to their immunogens. All of the clones appear to have binding affinities at or below nanomolar levels, which makes them strong candidates for future assay development.

5.2 Future Implications

This method of high-throughput screening can be applied to future work performed with hybridoma screening. As clones are developed, they can be ranked using SPR to rapidly identify antibodies that may be significant contributors to overall assay-development success. This methodology will minimize the time and labor that is normally spent on traditional enzyme-linked immunosorbent assay screening of hybridomas and allow for fast and informed decisions to determine which antibodies to use for future screening.

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ACRONYMS AND ABBREVIATIONS

OVA	ovalbumin from chicken egg
PBS	phosphate buffered saline
PBST	phosphate buffered saline/tween
RTB	ricin toxin B chain. subunit of ricin holotoxin
SPR	surface plasmon resonance

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APPENDIX A: LIGAND ANTIBODY CONCENTRATIONS AND CAPTURE LEVELS

Clone	Antibody Concentration (mg/mL)	*RL	Analyte
P1B7C12	0.09	266.7833	RTB
P1B7C2	0.08	270.3333	RTB
P1B7B12	0.05	226.1667	RTB
P6A7A8	0.09	292.5	RTB
P6A7E10	0.26	155.3333	RTB
P8G9F10	0.04	162.5	OVA
P8G9G7		564.5	OVA
P8G9C11	0.43	463.5	OVA
P8G9B10	2.2	627.5	OVA
P8G9F2	0.26	424.3333	OVA
P8G9A9		557.3333	OVA
P8G9G6		497	OVA
P8G9D10	0.08	317.8333	OVA
P8G9H1	0.15	264.5	OVA
P8G9C2	0.39	287	OVA
P8G9C10	0.2	228.3333	OVA
P8G9C9	1.86	517.5	OVA
P8G9F10	0.04	467.1667	OVA
P8G9H12	1.55	502.6667	OVA
P8G9G10	1.97	501.6667	OVA
P6A8G12	0.018	52	OVA
P6H8C6	0.12	240.6667	OVA
P6H8A2	0.058	34.5	OVA
P6H8A12	0.062	18.91667	OVA
P6H8H5	0.02	119.6667	OVA
P6H8E12	0.03	none	OVA
P8C4H10	0.2	380.1667	OVA
P8C4H9	0.59	476.5	OVA
P1D10B9	0.2	416.5	RTB
P1D10D12	0.85	483	RTB
P1D10E12	0.73	369.3333	RTB
P1D10C2	0.77	443.6667	RTB
P6A7D12		442.8333	RTB
P6A7G8	0.6	500.8333	RTB
P4A8F12		480.1667	RTB

Clone	Antibody Concentration (mg/mL)	*RL	Analyte
P4A8C12	1.4	491.8333	RTB
P4A8B5	0.77	417.3333	RTB
P4A8A12		561.1667	RTB
P4A8B9		536.8333	RTB
P4A8G5	2	628.8333	RTB
P3F4E4	0.77	533.8333	RTB
P1B4B8	1.6	430.1667	RTB
P4A8G4	0.16	339.1667	RTB
P4A8G9	0.5	447.5	RTB
P1D10B11	1.3	551.6667	RTB
P3F4F7	0.92	513	RTB
P1B7C12	0.09	357.6667	Ricin holotoxin
P1B7C2	0.08	351	Ricin holotoxin
P1B7B12	0.05	294.1667	Ricin holotoxin
P6A7A8	0.09	383.8333	Ricin holotoxin
P6A7E10	0.26	260.5	Ricin holotoxin
P1D10B9	0.2	448.1667	Ricin holotoxin
P1D10D12	0.85	562	Ricin holotoxin
P1D10E12	0.73	399.6667	Ricin holotoxin
P1D10C2	0.77	520.8333	Ricin holotoxin
P6A7D12		526.5	Ricin holotoxin
P6A7G8	0.6	543.6667	Ricin holotoxin
P4A8F12		519.3333	Ricin holotoxin
P4A8C12	1.4	523.8333	Ricin holotoxin
P4A8B5	0.77	463.3333	Ricin holotoxin
P4A8A12		633.3333	Ricin holotoxin
P4A8B9		520.8333	Ricin holotoxin
P4A8G5	2	603.6667	Ricin holotoxin
P3F4E4	0.77	487.1667	Ricin holotoxin
P1B4B8	1.6	417.5	Ricin holotoxin
P4A8G4	0.16	331.5	Ricin holotoxin
P4A8G9	0.5	452.1667	Ricin holotoxin
P1D10B11	1.3	542.8333	Ricin holotoxin
P3F4F7	0.92	522.5	Ricin holotoxin

*RL: the response units of ligand captured on a chip surface.

APPENDIX B: LANGMUIR 1:1 FIT OF OVALBUMIN (OVA) CLONES WITH OVA

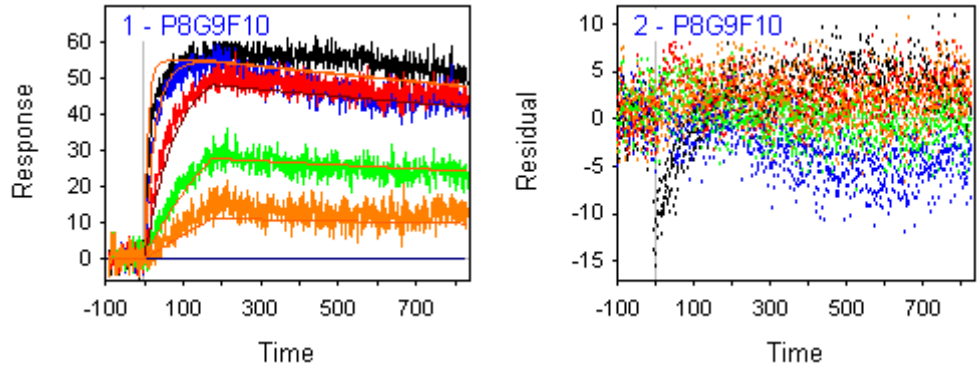

Figure B-1. Langmuir 1:1 fit of clone P8G9F10 with OVA.

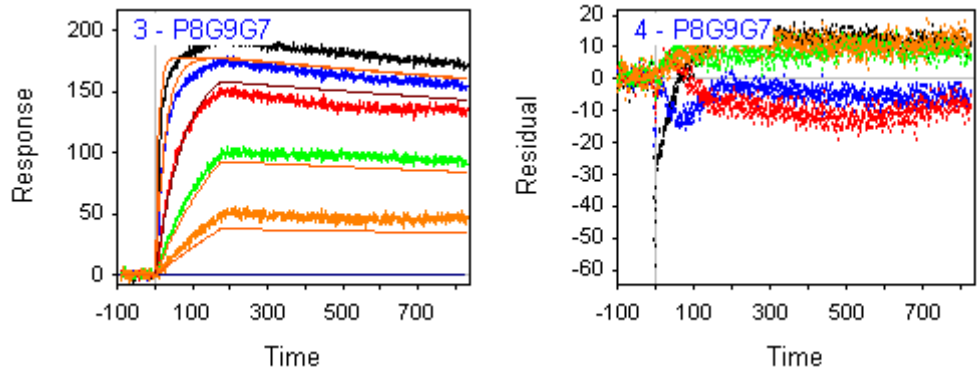

Figure B-2. Langmuir 1:1 fit of clone P8G9G7 with OVA.

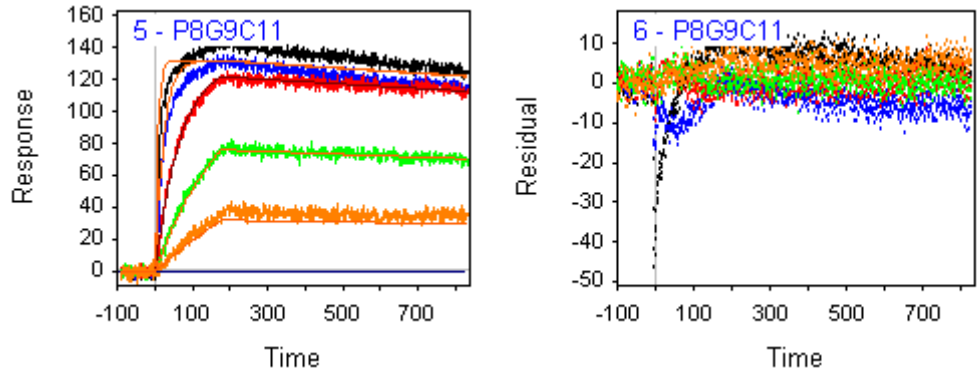

Figure B-3. Langmuir 1:1 fit of clone P8G9C11 with OVA.

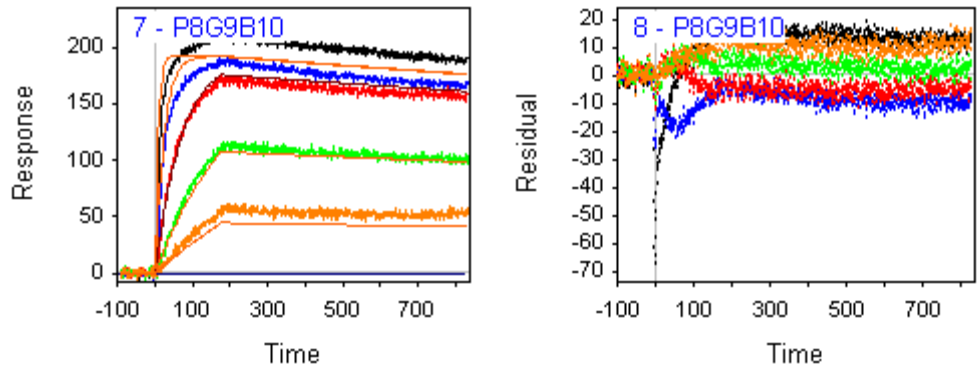

Figure B-4. Langmuir 1:1 fit of clone P8G9B10 with OVA.

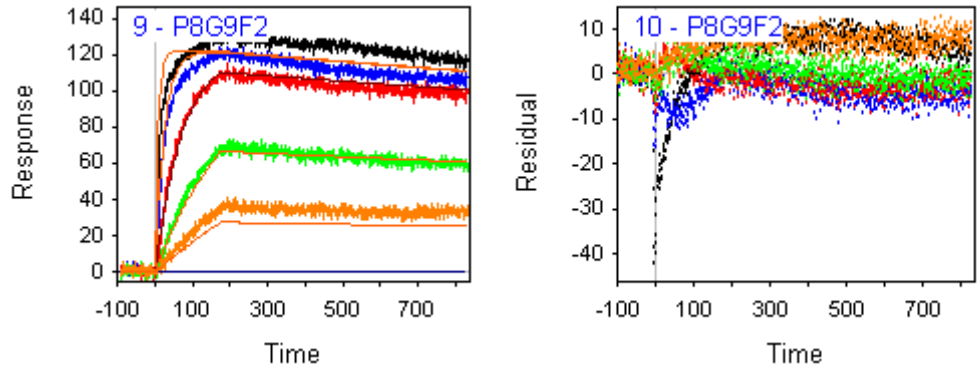

Figure B-5. Langmuir 1:1 fit of clone P8G9F2 with OVA.

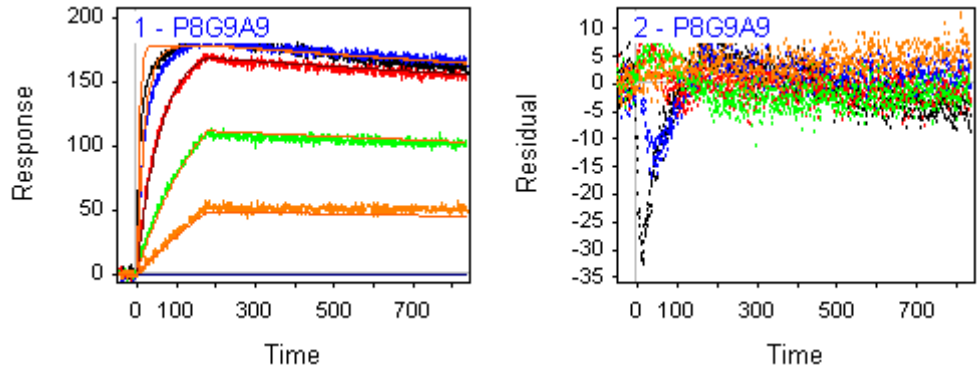

Figure B-6. Langmuir 1:1 fit of clone P8G9A9 with OVA.

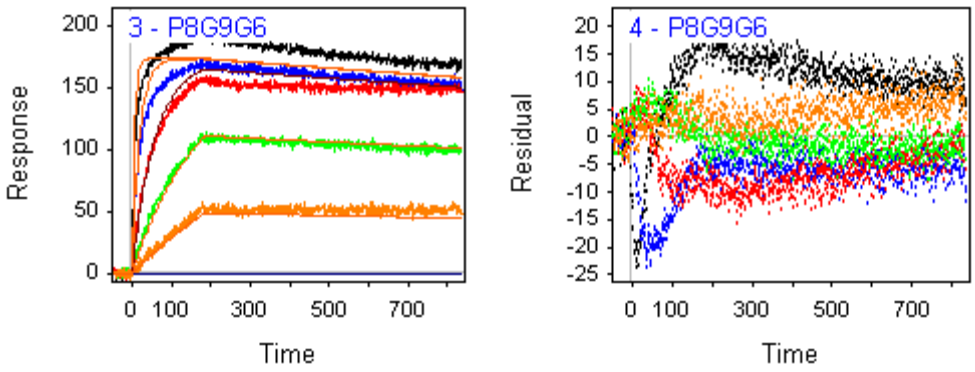

Figure B-7. Langmuir 1:1 fit of clone P8G9G6 with OVA.

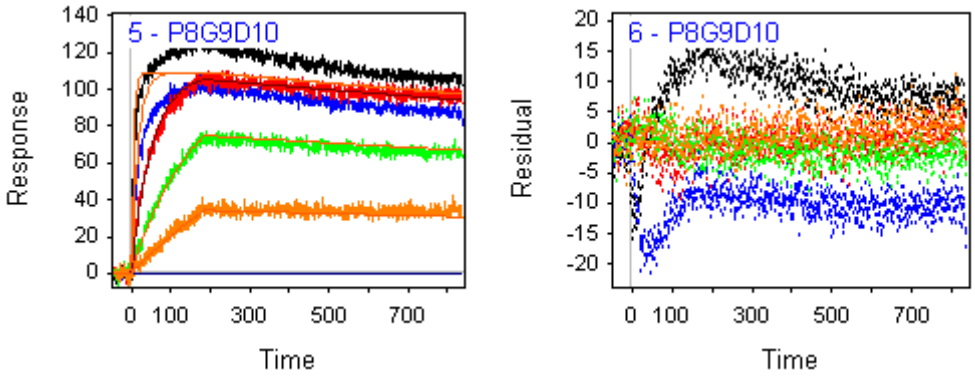

Figure B-8. Langmuir 1:1 fit of clone P8G9D10 with OVA.

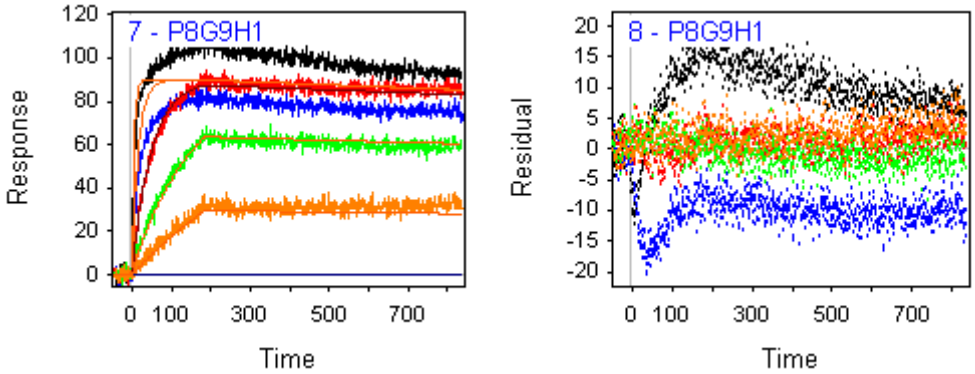

Figure B-9. Langmuir 1:1 fit of clone P8G9H1 with OVA.

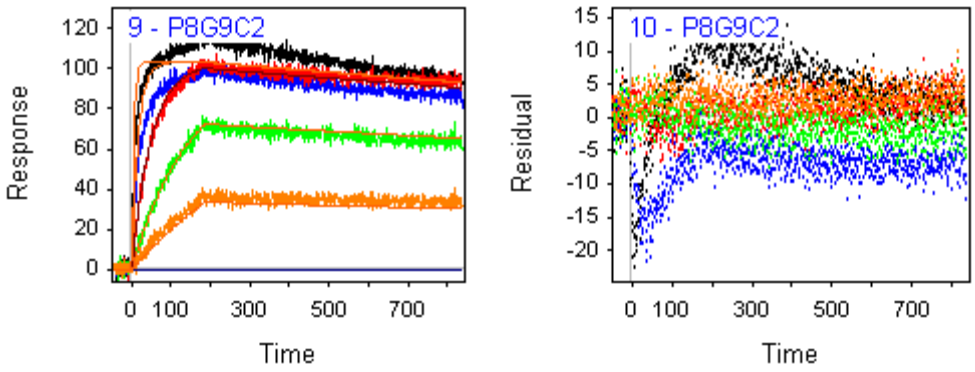

Figure B-10. Langmuir 1:1 fit of clone P8G9C2 with OVA.

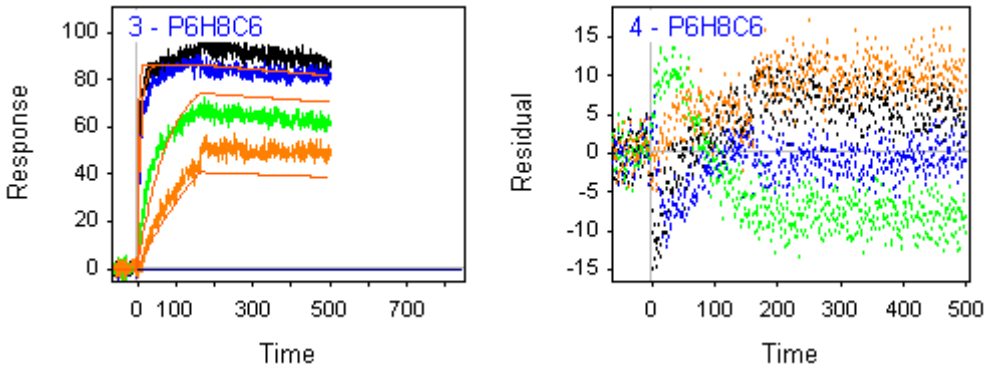

Figure B-11. Langmuir 1:1 fit of clone P6H8C6 with OVA.

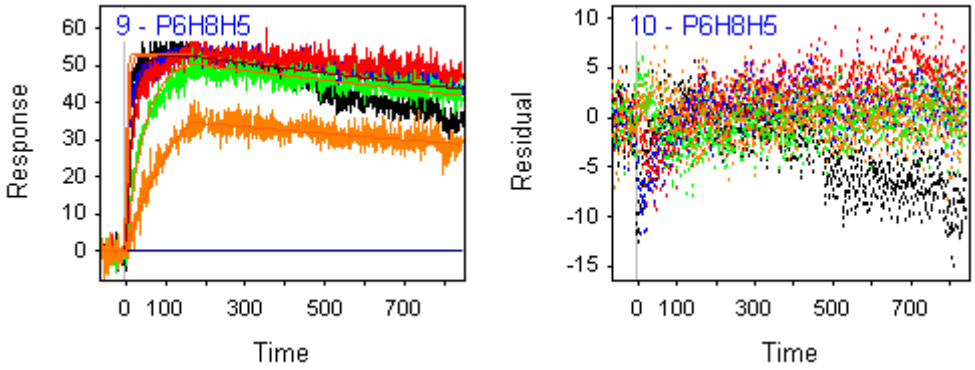

Figure B-12. Langmuir 1:1 fit of clone P6H8H5 with OVA.

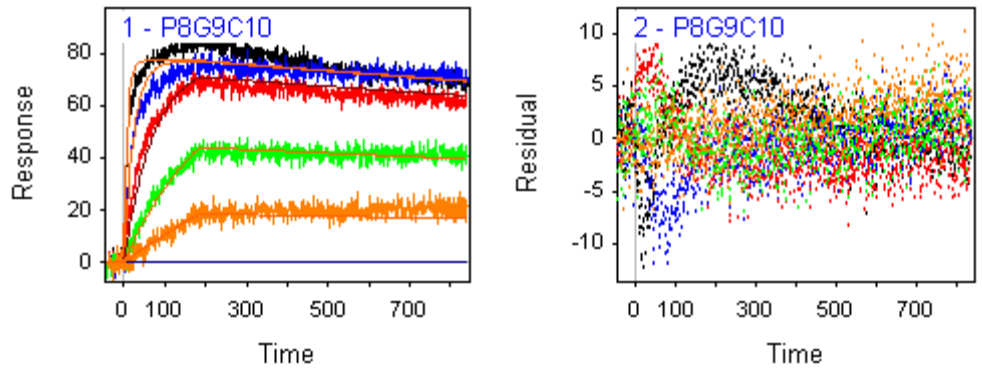

Figure B-13. Langmuir 1:1 fit of clone P8G9C10 with OVA.

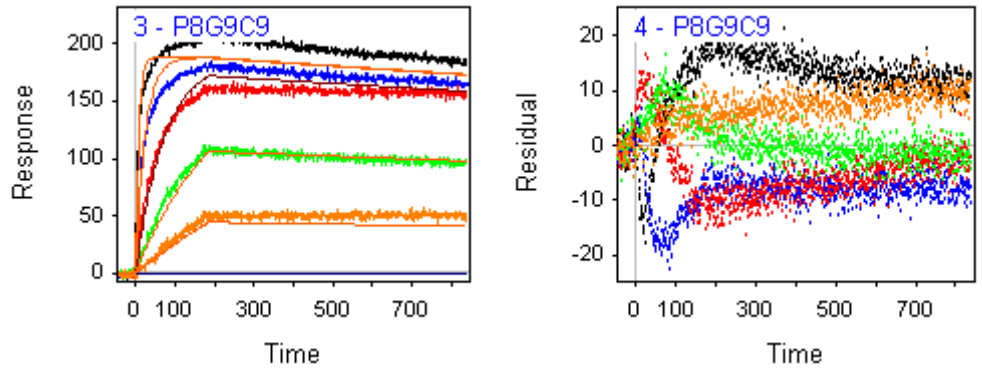

Figure B-14. Langmuir 1:1 fit of clone P8G9C9 with OVA.

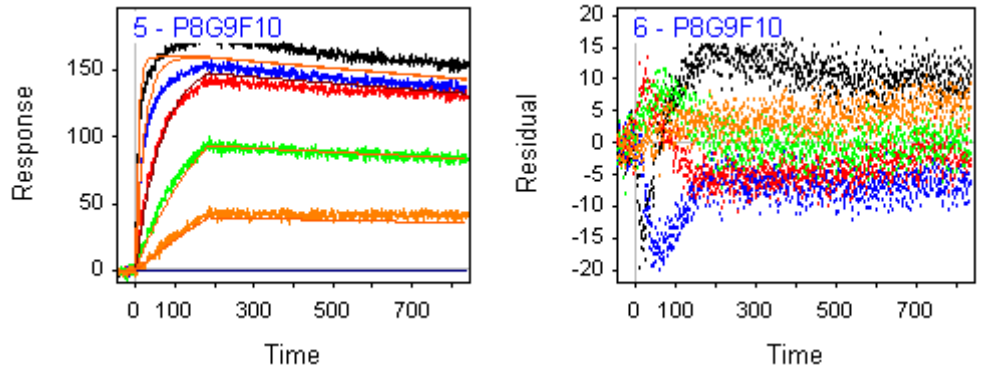

Figure B-15. Langmuir 1:1 fit of clone P8G9F10 with OVA.

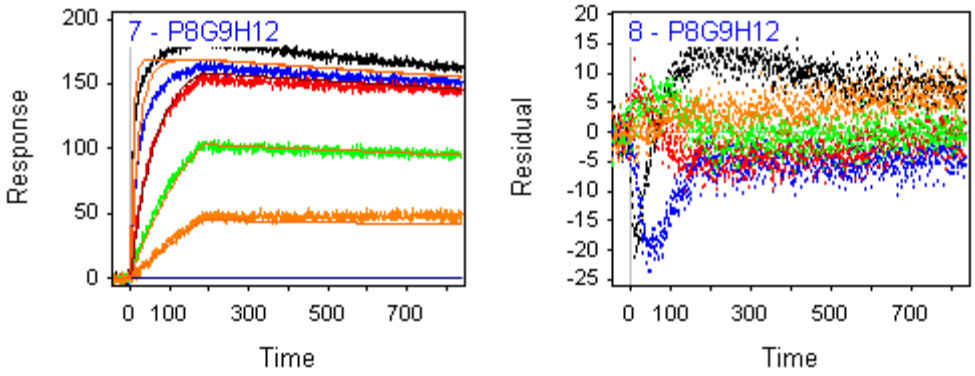

Figure B-16. Langmuir 1:1 fit of clone P8G9H12 with OVA.

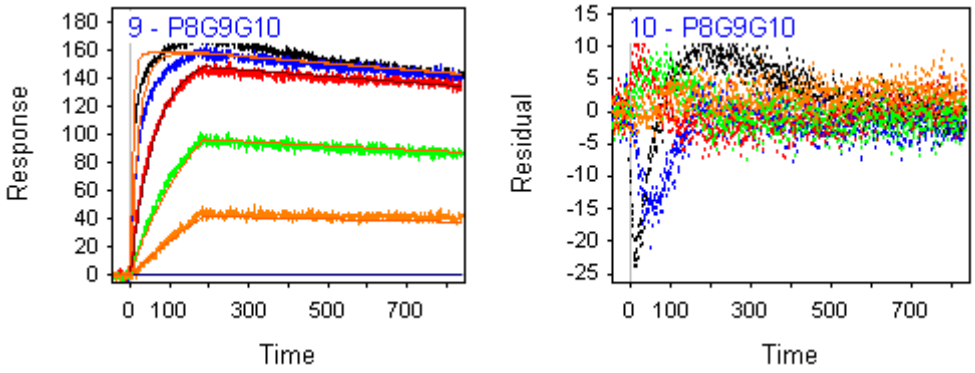

Figure B-17. Langmuir 1:1 fit of clone P8G9G10 with OVA.

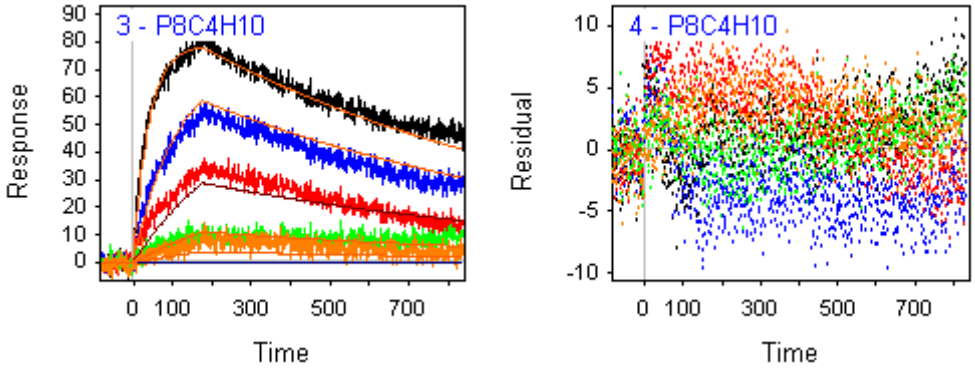

Figure B-18. Langmuir 1:1 fit of clone P8C4H10 with OVA.

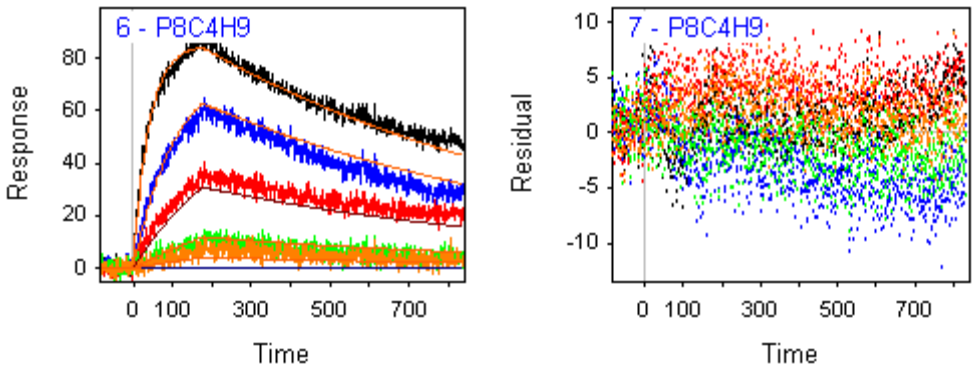

Figure B-19. Langmuir 1:1 fit of clone P8C4H9 with OVA.

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APPENDIX C: LANGMUIR 1:1 FIT OF RICIN TOXIN B (RTB) CLONES WITH RTB CHAIN

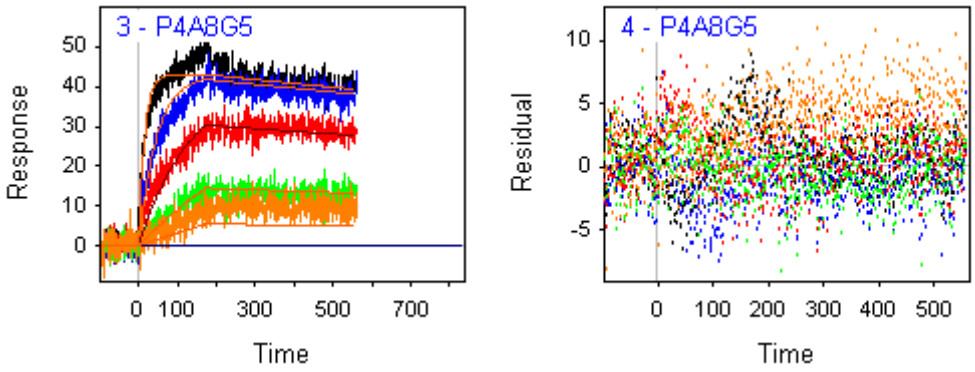

Figure C-1. Langmuir 1:1 fit of clone P4A8G5 with RTB.

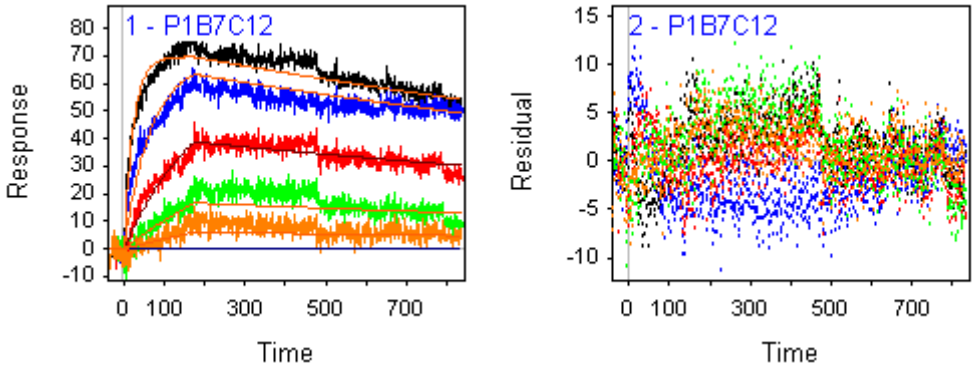

Figure C-2. Langmuir 1:1 fit of clone P1B7C12 with RTB.

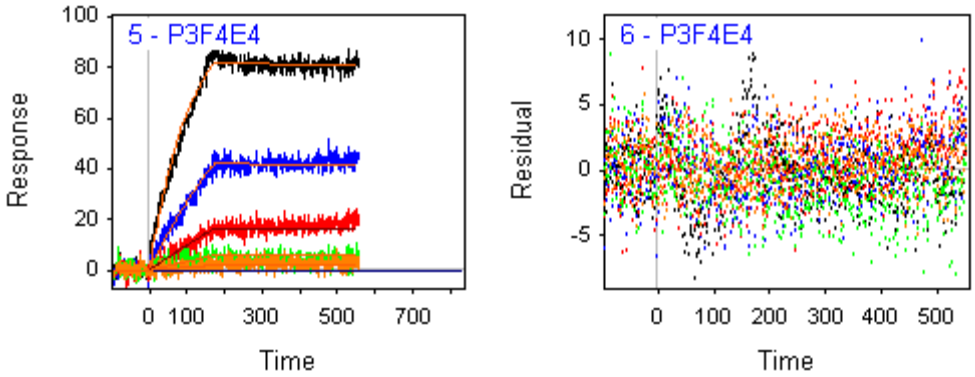

Figure C-3. Langmuir 1:1 fit of clone P3F4E4 with RTB.

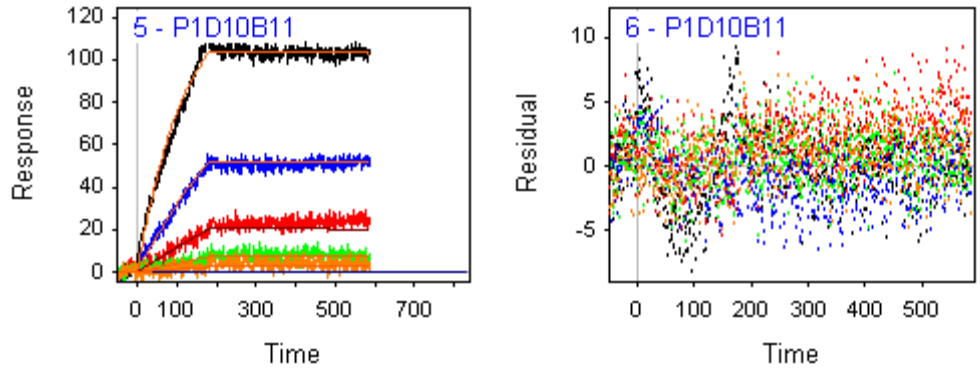

Figure C-4. Langmuir 1:1 fit of clone P1D10B11 with RTB.

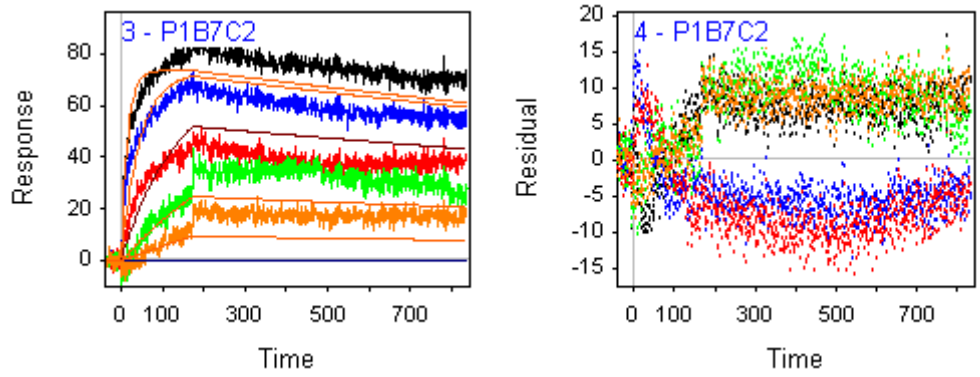

Figure C-5. Langmuir 1:1 fit of clone P1B7C2 with RTB.

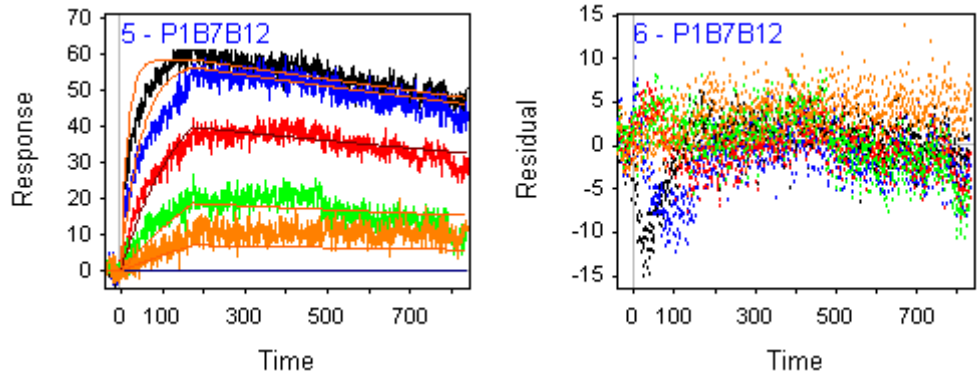

Figure C-6. Langmuir 1:1 fit of clone P1B7B12 with RTB.

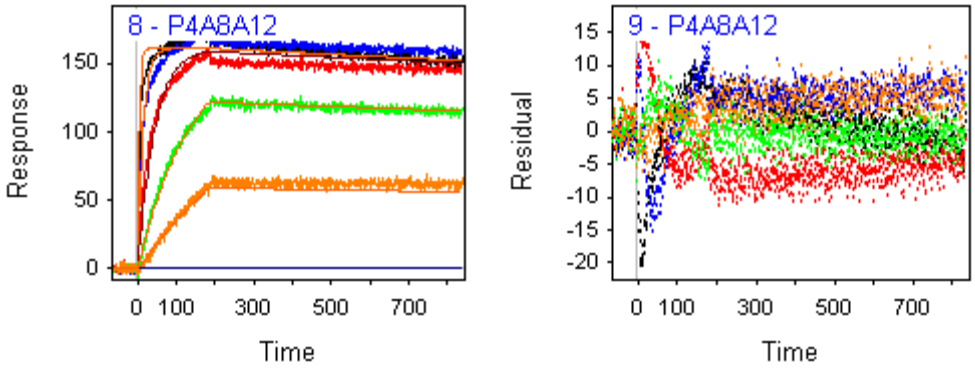

Figure C-7. Langmuir 1:1 fit of clone P4A8A12 with RTB.

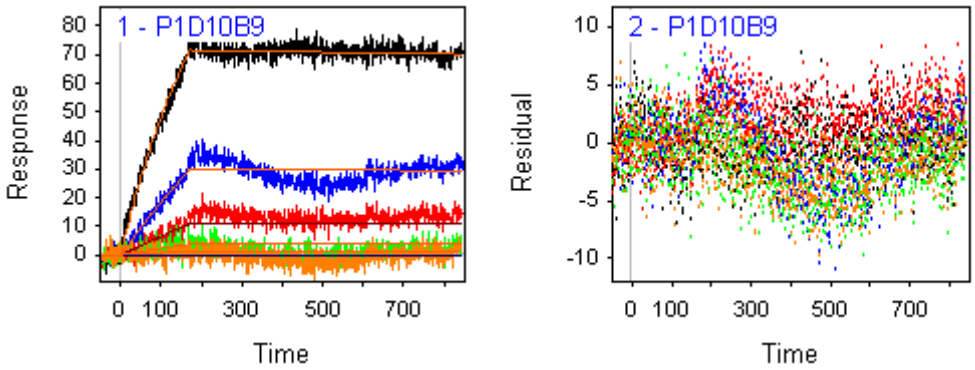

Figure C-8. Langmuir 1:1 fit of clone P1D10B9 with RTB.

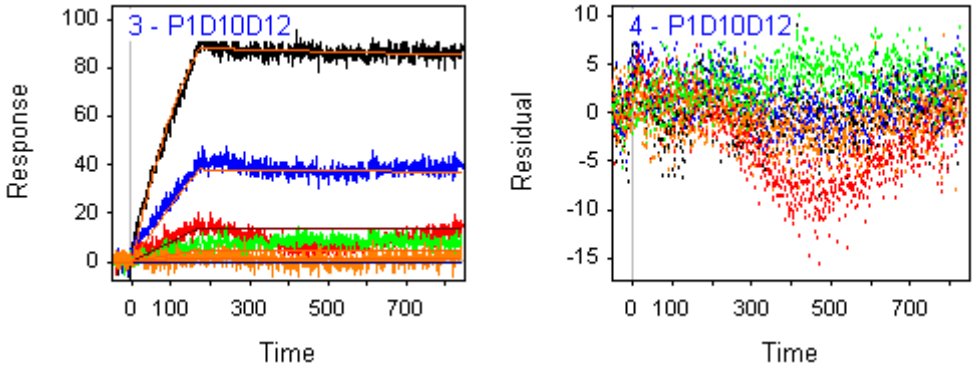

Figure C-9. Langmuir 1:1 fit of clone P1D10D12 with RTB.

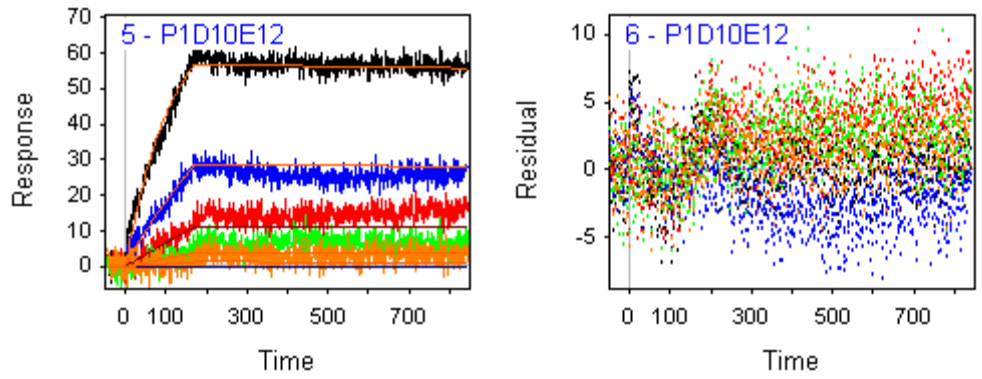

Figure C-10. Langmuir 1:1 fit of clone P1D10E12 with RTB.

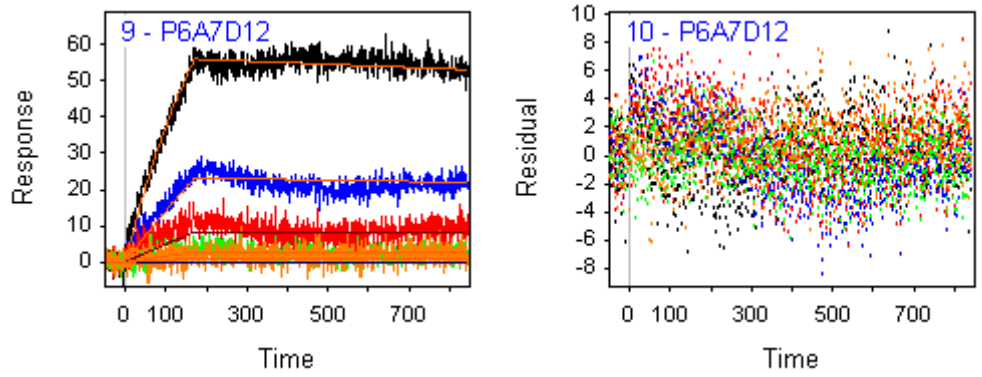

Figure C-11. Langmuir 1:1 fit of clone P6A7D12 with RTB.

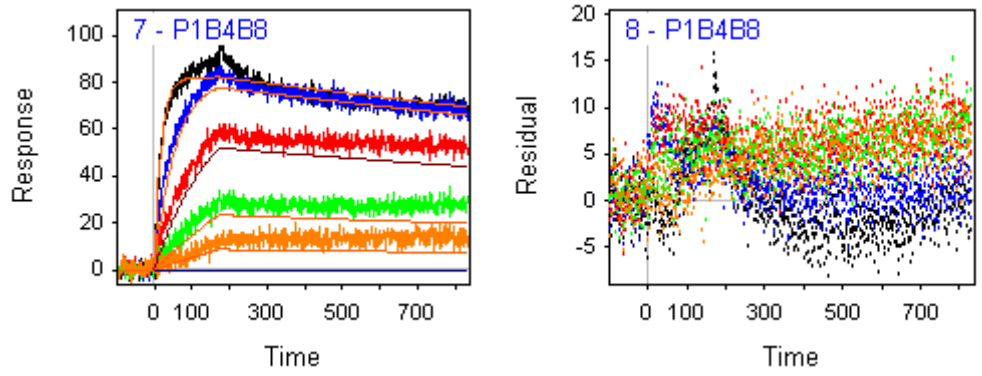

Figure C-12. Langmuir 1:1 fit of clone P1B4B8 with RTB.

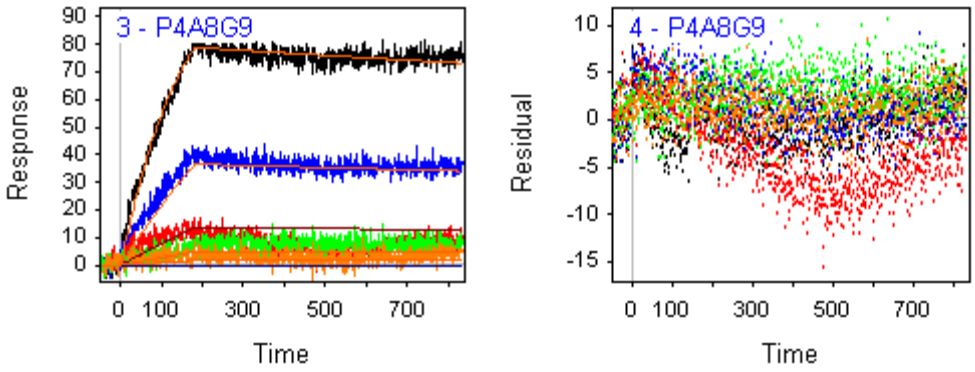

Figure C-13. Langmuir 1:1 fit of clone P4A8G9 with RTB.

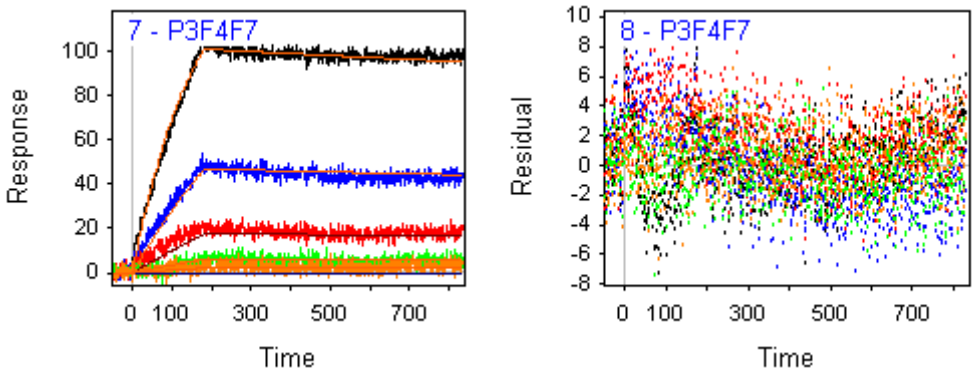

Figure C-14. Langmuir 1:1 fit of clone P3F4F7 with RTB.


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